Arizona Iceberg Lettuce Research Council
Project Annual Report: July 2005 – September 2006

Project Title: Detection of *Fusarium oxysporum* f. sp. *lactucae* in lettuce seed and soil

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Research Locations: Department of Plant Pathology, University of Arizona, Tucson and various fields in the winter lettuce producing area around Yuma, AZ.

Purpose of the Study

In 2001, the disease Fusarium wilt of lettuce, caused by the fungus *Fusarium oxysporum* f.sp. *lactucae*, appeared in 5 fields near Wellton, Arizona. In 2002, the disease was reported in additional fields in the Gila Valley and near Yuma, and it became apparent that the pathogen was spreading in the prime winter lettuce growing region of Arizona. Because this a relative new disease, very little is known about the etiology and epidemiology of Fusarium wilt, and few options are currently available to growers to manage it. Therefore, a comprehensive research program was initiated in order to develop basic understanding of the biology and genetics of the fungus. In this study, several objectives have been developed over four years of funding. These include:

1. Develop DNA-based pathogenic PCR primers to detect the pathogen and not other related species
2. Develop a seed assay to detect the pathogen in seed
3. Screen commercial seed for the presence of the pathogen
4. Perform greenhouse-based studies to investigate the production of infested seed from infected lettuce plants
5. Develop a soil assay to detect the pathogen in field soil (and also in lettuce tissue)
6. Examine population genetic diversity of the pathogen to understand movement within Arizona and between California and Arizona

Objective 1, 2, 3, 4, and 6 have all been successfully initiated and over the years progress has been significant to the point where the objectives are complete and results are currently being prepared for publication in peer-reviewed journals. Objective 5 has consistently given us problems and results to date are not where we had hoped to be at the onset. Nonetheless, overall the project has been very successful and we now know a considerable amount regarding the phylogenetics of *Fusarium oxysporum* f.sp. *lactucae* (where did it come from), have developed a very sensitive method to detect it in commercial seed (how can we detect it), its ecological potential for contaminating commercial lettuce seed (can it be seedborne), its occurrence in commercial lettuce seed based upon random sampling (is it an obvious and significant problem

in seed), and the distribution of genotypes in Arizona and California (how different are populations in each state). At this time, our research is at a point where we can propose several recommendations.

1. All evidence suggests that long-distance spread of *Fol* is via commercial lettuce seed. We have developed a very sensitive and specific test for the presence of *Fol* on seed. Thus, this method needs now to be implemented by commercial seed testing institutions for service to the lettuce industry. The demand for such a service by those in the lettuce industry needs to be clear and direct if the commercial seed testing institutions are to respond to the request.

2. *Fo*l can contaminate commercial seed. This is most likely via contact with infected crop residue, although evidence also shows that direct infection is possible at a very reduced frequency. All seed crops need to be rigorously examined before harvest for any sign of Fusarium wilt. However, our studies reveal that symptoms may not be evident for many cultivars. Thus, a simple field evaluation may not be sufficient for effective assessment. If possible, indicator cultivars may be included in any seed crop that would readily succumb to the pathogen. In addition, all fields with a history of Fusarium wilt must be avoided. Moreover, as contamination occurs readily upon exposure to infested crop residue, all seed processing equipment needs to be rigorously cleaned between seed lots to prevent potential cross contamination. This is especially true in seed production areas where Fusarium wilt has been documented, e.g., the Huron area of California.

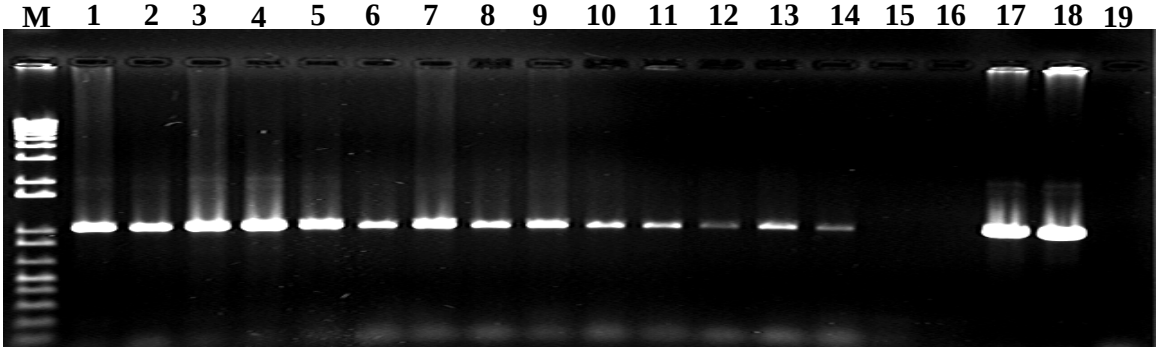
3. Fol population are varied across California and, to a limited extent, in Arizona. These population fingerprints can be used to monitor spread of the pathogen and detect possible introduction of new genotypes, as well as new races. Therefore, continued monitoring of newly infested fields should be maintained to establish baselines for local diversity. The data may not have immediate utility, but will provide a genetic history of the region and be invaluable when significant shift in population diversity occurs.

Objectives of our 2005-2006 research.
1. Validation of current seed testing protocol.
2. Survey of commercial seed lots supplied to UofA.
3. Genetic fingerprinting of new isolates obtained from infested fields.
4. Optimization of DNA-based soil assay.

Results.
1. *Validation of current seed testing protocol.*
In 2005-2006, seed lots determined pathogen free, were spiked with seed that was artificially contaminated with Fol. Levels of infestation ranged from 100% to 0.1%. All infestation, DNA extraction, and PCR detection was performed in dublicate. Results revealed that the seed assay method can reliable detect seed infestation in lots with infestation levels as low as 0.1% (Fig. 1). No false positives were detected in this validation test. This in itself is a significant improvement over previous versions of the test in which false positive were a recurrent problem.

The reduction in the incidence of false positive is a result of improvements in PCR primers and a simplification of protocol which eliminated a problematic DNA ligation step. As such, the current protocol for testing seed for *Fol* infestation is robust and ready for more routine application. A manuscript is currently in preparation that will present this method of seed testing to the scientific community and to industry.

Figure 1. Detection of *F. oxysporum* f. sp. *lactucae* from lettuce seeds having different levels of artificial infestation with the nested PCR-based assay. Lanes 1 to 16 show results from two independent DNA extractions for each seed lot having the indicated rates of infestation: Lanes 1 and 2, 100%; lanes 3 and 4, 10%; lanes 5 and 6, 5%; lanes 7 and 8, 1%; lanes 9 and 10, 0.5%; lanes 11 and 12, 0.2%; lanes 13 and 14, 0.1%; and lanes 15 and 16, 0%. Lanes 17 and 18 are the positive controls (genomic DNA from mycelia). Lane 19 is the negative control (no DNA) and lane M contains the 1-kb plus ladder.



2. *Survey of commercial seed lots supplied to UofA.*

In 2005-2006, 18 seed lots were supplied by local and regional seed companies for analysis for the presence of *F. o.* f. sp. *lactucae.* All seed was raw (uncoated) and was supplied to us with anonymous lot numbers. For each seed lot, 5000 seeds from each lot were subjected to the PCR-based method of detection developed in our lab. In addition, 1500 seeds were plated on *Fusarium* selective media and examined after 7 days incubation. Following incubation, suspect *Fol* colonies were subcultured and tested for pathogenicity in greenhouse studies.

All seed lots tested were negative for *Fol* based upon the PCR-based method. Results of the direct plating method resulted in 11 suspect *Fol* colonies (Table 1). None of the suspect colonies proved to be pathogenic based upon our pathogenicity tests.Since the initiation of this project in 2002, 88 seed lots have been examined for the presence of *F. o.* f. sp. *lactucae.* None have been positive to date.

Table 1. Evaluation of the presence of *F. o.* f. sp. *lactucae* in 18 lettuce seeds lots randomly sampled from commercial seed growers in Arizona in 2006.

Lettuce seed lot	Number of *F. oxysporum* colonies isolated/number of seeds plated	*F. oxysporum* isolate code
# 1	0/1500	-
# 2	0/1500	-
# 3	0/1500	-
# 4	1/1500	FoS4
# 5	0/1500	-
# 6	0/1500	-
S15	0/1500	-
22-414	0/1500	-
22-214	0/1500	-
SJJ	0/1500	-
SJJ1	0/1500	-
RLAH	2/1500	RLAH1; RLAH2
IHG	0/1500	-
IHF	0/1500	-
RHD	6/1500	RHD1; RHD2; RHD3; RHD4; RHD5; RHD6
IHC	0/1500	-
RLHB	2/1500	RLHB1; RLHB2
RHA	0/1500	-

3. Genetic fingerprinting of new isolates obtained from infested fields. In 2005-2006, six additional *Fol* isolates were obtained from six newly infested fields. This bring the total number of isolates obtained from the Yuma area to 40. Note that this does not mean 40 fields are infested as some fields have provided more than one isolate. Using our established technique known as microsatellite-primed PCR (MP-PCR), we generated DNA fingerprints from these isolates and compared them with isolates previously obtained from both Arizona and California. Although only six newly infested fields were reported to UA personnel during 2005-2006, it is believed that a number of other fields were newly infested and reported only to PCAs. This is a significant impediment to documenting the full extent of *Fol* infestation in the Yuma area.

Of these six isolates, two represented new genotypes and four represented the most common genotype found throughout the Yuma area (Table 2). As observed, the distribution of lactucae haplotypes in AZ fits three patterns: a single haplotype of high frequency (1-05), a single haplotype shared by two fields (I-01), and the rest appearing in single fields. This distribution suggests more than one independent introductions of the pathogen into AZ. Although the results are skewed by the low sample representation per field and per year, they

establish a baseline distribution and a more representative sampling of isolates in the future may give a better representation of the haplotypes within AZ. Equally important, even though new haplotypes were observed in Arizona only after 2003, this does not preclude the possibility that these haplotypes were present in Arizona before that time and became evident only at a later date.

The California isolate population reflects a greater genetic diversity than the Arizona isolates with a total of 23 haplotypes represented. The majority of these haplotypes were found within two major clusters (Fig. 2). The data reported here may also provide an indication of where the f. sp. *lactucae* was first established in the United States. Although it is well known that the original site of diagnosis is not necessarily the site of origin, in this case, the fact that the disease was first diagnosed in California and that California contains a high level of diversity strongly supports this state as being the site of origin. A similar study on genetic diversity of Fol is being conducted by Dr. Tom Gordon of UC Davis using a slightly different fingerprinting technique. Our results and those of Dr. Gordon's will be combined in a single analysis of Fol diversity across Arizona and California, and will be published later this year. A copy of that publication will be forwarded to both the Arizona and California lettuce boards.

Table 2. The evolution of haplotypes in Arizona over a period of five years indicates that there are presently seven different haplotypes in Arizona.

Year	Genotype	# of Fields	Primers						
			1	2	3	4	5	6	7
2001	1	24	A	A	A	A	A	A	A
2002	1	03	A	A	A	A	A	A	A
2003	2	08	A	A	A	A	A	A	A
			A	A	A	A	B	B	A
2004	5	05	A	A	A	A	A	A	A
			B	A	A	B	C	A	A
			A	A	A	A	B	B	A
			A	A	A	B	C	A	A
			B	B	C	C	E	C	B
2005	3	06	A	A	A	A	A	A	A
			A	A	A	B	C	D	A
			C	A	A	B	C	D	A

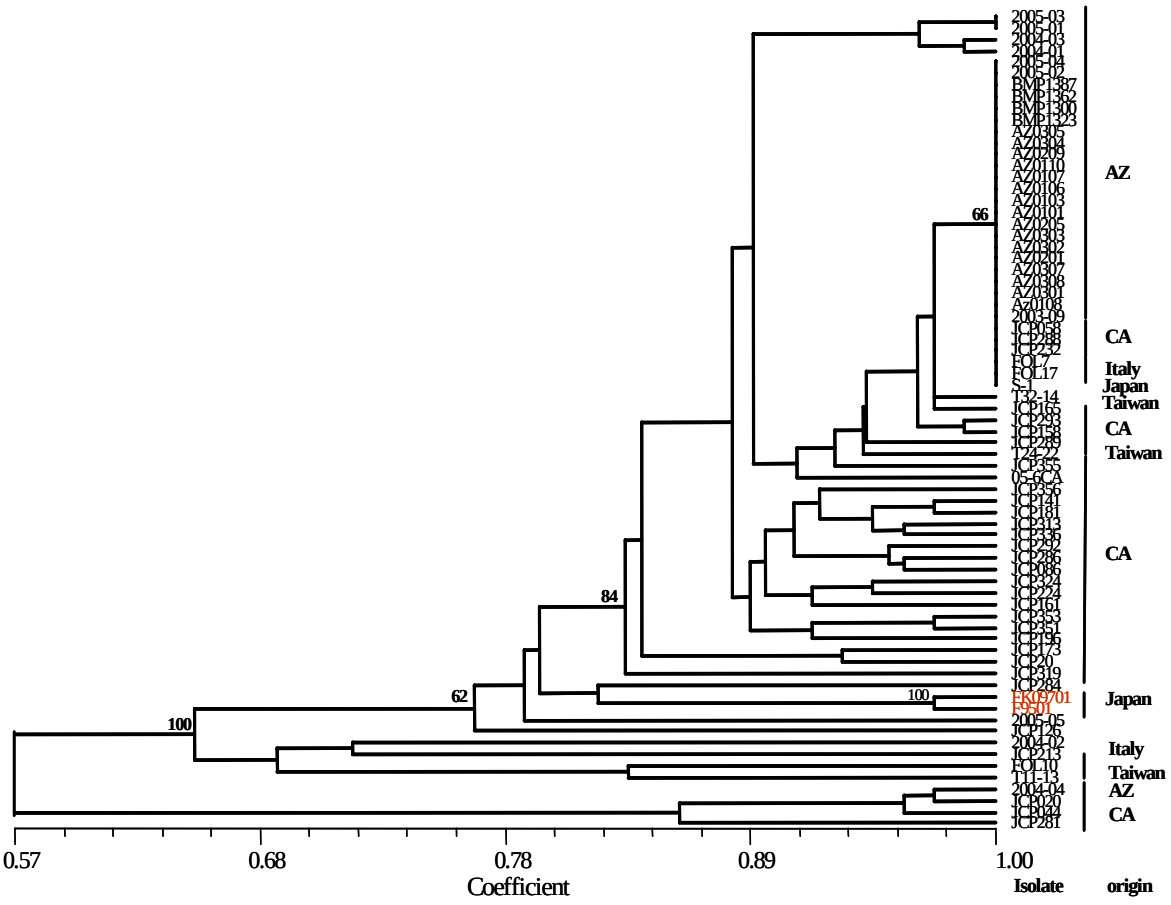


Figure 2. Dendrogram of combine data of *F. oxysporum* isolates collected from Arizona and California, as well as several other locations around the world, using ISSR primers $GACA_4$, GTG_5, and the minisatellite primers M13 and T3B. Six *F. oxysporum* nonpathogenic to lettuce were included for comparison. Isolates of *F. oxysporum* f. sp. race 2 are presented in red. Numbers on branches represent bootstrap values greater than 50% based on 1000 replicates. The bottom scale is the percentage of similarity by Jaccard's similarity coefficient. The figure reveals the high diversity found in California and the limited diversity found in Arizona.

4. Optimization of DNA-based soil assay. Efforts have been made over several years to successfully amplify *Fol* DNA from samples of soil collected from fields with documented incidence of Fusarium wilt of lettuce. From the initial attempts with this technique, problems were encountered with the PCR caused by substances in the soil that inhibit the enzyme needed for the PCR amplification reaction to occur. Over 8 different protocols for DNA extraction and purification have been tested in modified protocols to removed potential contaminants in the soil DNA extract that either directly inhibit the enzyme or bind with the Mg++ needed for the reaction to occur.

The efforts were successful in allowing amplification of fungal DNA from soil samples which were spiked with very high amount of DNA as an amplification template (>10 ng DNA/ul

of sample). These results have been reported in previous AILRC annual reports. However, to this date we have not been able to successfully amplify low quantities of spiked DNA in soil samples (< 10 ng DNA/ul of sample), which may be similar to levels found in infested fields. Moreover, DNA extracts from soil samples recovered from Arizona fields in which Fusarium wilt was at a very high incidence, failed to serve as suitable templates for PCR amplification (i.e., no *Fusarium* was detected). That these field soil samples did in fact contain the *Fol* pathogen was confirmed by successfully detecting the fungus using standard soil dilution plating on semi-selective growth medium.

Thus, we have been successful in using our PCR detection technique for detection of *Fol* from samples of seed and lettuce tissue, but not from naturally infested field soil. However, new techniques are constantly emerging that increase efficacy at 1) recovering minute amounts of DNA from environmental samples, and 2) improving the removal of contaminating substances that inhibit the enzymatic reaction necessary for successful PCR. In other words, although we have not yet been successful in this effort, future efforts may in fact bring about more encouraging results and the eventual application of PCR technology to *Fol* detection in samples of infested field soil. Our lab is dedicated to pursuing this goal.